Differences in the Ligand Specificity between CD1d-Restricted T Cells with Limited and Diverse T-Cell Receptor Repertoire

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The natural killer (NK) T-lymphocyte population consists of two subsets utilizing a diverse and restricted T-cell receptor (TCR) repertoire, respectively. Both populations have been shown to include autoreactive cells. NKT cells carrying restricted Vα14(AV14S1)Jα281/Vβ8.2(BV8S2A1) TCR have been shown to recognize α-galactosylceramide (αGalCer) presented in the context of murine CD1d. In this study we screened a set of murine CD1d-autoreactive T-cell hybridomas with diverse TCR for their reactivity with several glycosylated variants of ceramide, including αGalCer. These hybridomas showed a different pattern of reactivity to CD1d-expressing antigen-presenting cells (APC) and were not reactive with any of the tested variants of ceramide. A second set of hybridomas had been selected for expression of Vα14 and Vβ8.2 TCR chains. These cells responded to αGalCer presented on CD1d, but were only weakly reactive to syngeneic splenocytes or CD1d-transfected cells. Their fine specificity in the response to glycosylation variants of ceramide demonstrated a homogenous reactivity pattern, including reactivity to α-galactosylsphingosine, the variant of αGalCer with truncated fatty acyl chain. These findings underline the differences in ligand specificity between the two subsets of CD1d-restricted NKT cells, and demonstrate a similarity in reactivity among the hybridomas using the Vα14-Jα281/Vβ8.2 TCR.

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INTRODUCTION

Natural killer (NK) T cells (NKT) represent a unique subset, which differs from the conventional T cells by the expression of activation markers and markers characteristic for NK cells (NK1.1, CD122, CD16 and members of the Ly49 family) [1], and reactivity to the major histocompatibility complex (MHC) class I-like molecule CD1 [2]. The cells are present at low percentage in most lymphoid organs, i.e. the thymus, spleen and lymph nodes and make up 20–30% of all T cells in the liver and bone marrow. The majority of NKT cells are either double negative (DN) or $CD4^+$. The immunological function of the NKT-cell population has not been clarified yet. They have been postulated to play a role in the induction of T helper (Th) type 2 immune responses [3–6], in the regulation of autoimmunity [7–9], in defense against bacterial infections [10, 11], and participate in the clearance of liver metastases [12]. They are further reported to act as T helper cells for B cells during the immunoglobulin (Ig) G responses against glycosylphosphatidylinositol (GPI)-anchored antigens from *Plasmodium* and *Trypanosoma* [13].

Murine NKT cells have been shown to preferentially use an invariant Vα14(AV14S1)-Jα281 TCR α-chain, which is combined with a β-chain using one of the Vβ8(BV8S1), Vβ2(BV9S1) or Vβ7(BV12S1T) segments [14]. In addition there is a second subset of CD1d-reactive cells, which has a diverse TCR repertoire. These cells were first identified as a subset of the $CD4^+$ T-cell population found in the periphery of MHC class II-deficient mice [15]. The cells are thought to share some similarities with $V\alpha14^+$ NKT cells, notably the memory phenotype, the ability to produce high amounts of interleukin (IL)-4 and interferon (IFN)-γ upon activation and CD1d-restriction (M. Sköld and S. Cardell, unpublished observation). A similar population was later found in normal C57BL/6 [16] and in BALB/c mice [17]. Studies on β2-microglobulin (β2m)-deficient and later on CD1-deficient mice demonstrated that CD1d is required for the development of the majority of NKT cells [18–22].

The CD1 family is referred to as a third lineage of antigen-presenting molecules, found in many species including human and rodents. Sequence comparison reveals that they can be divided into two groups; group one consisting of humans CD1a, b, c and group two including human CD1d and both murine CD1 molecules, CD1d1 and CD1d2. The murine CD1d is expressed on several cell types of the hematopoietic lineage [23], while human group one CD1 molecules have a more restricted expression pattern [24]. Human CD1b and CD1c molecules can present several types of lipoglycan antigens to T cells [25–28], while murine CD1d can also present hydrophobic peptides to T cells [29]. The nature of the endogenous CD1d-associated ligands recognized by the autoreactive NKT cells is still unclear, however, GPI was identified as a major natural ligand presented on CD1d in murine cells [30]. CD1d is able to present synthetic glycolipid antigens such as α-galactosylceramide (αGalCer) to NKT cells using the Vα14$^+$ type of TCR [31–33]. αGalCer was originally purified from a marine sponge and so far such compound or a homologue has not been found in mammalian tissues. Although αGalCer is a common ligand for most of Vα14$^+$ NKT cells, recent studies have demonstrated distinctions in ligand preference between different Vα14$^+$ NKT cells [34]. To date it is unclear, what type of ligand(s) can activate NKT cells that display diverse TCR. Some studies imply a heterogeneity in antigen specificity within this population of CD1-autoreactive T cells [15, 35–37]. On the other hand, the highly conserved and essentially nonpolymorphic nature of the CD1 molecule might suggest that both murine CD1d-restricted subpopulations of NKT cells recognize similar ligands. In this report we have analyzed NKT cells with diverse TCR by screening them for the reactivity to a panel of various glycosylated ceramides presented on murine CD1d. Further, we examined the differences in fine specificity within a panel of the Vα14$^+$ hybridomas.

MATERIALS AND METHODS

Cell lines and hybridoma cells with diverse TCR. The RMA-S cells transfected with murine CD1d (mCD1) were kindly provided by Dr Mitchell Kronenberg (Division of Developmental Immunology, La Jolla Institute of Allergy and Immunology, San Diego, USA). CD1d transfected A20 cells were obtained in the laboratory by electroporation of mCD1 construct from Dr Mitchell Kronenberg, L cells transfected with mCD1 were described before [15]. The CD1d-transfectants were cultured in RPMI 1640 from Gibco BRL (Life Technologies, Paisley, UK), supplemented with 10% FCS (Sigma Cell Culture, St. Louise, CA, USA), 100 U/ml penicillin/streptomycin, 1 mM sodium pyruvate, 50 mM of 2-mercaptoetanol (Gibco BRL). In order to analyze the CD1 levels on the cells, the CD1d-transfectant cells were stained with a biontinylated anti-CD1 (1B1) antibody (Ab) [23] and with a biotinylated isotype matched control Ab, followed by PE-conjugated streptavidin (Southern Biotechnology Associates, Inc., Birmingham, AL, USA). The CD1d-reactive VIII24, XV19, VII57, IIX82, XV104 hybridoma cells with diverse, non-Vα14 TCR originate from CD4$^+$ cells from MHC class II-deficient mice as described before [15].

Vα14$^+$Vβ8.2$^+$ T-cell hybridomas. Vα14$^+$Vβ8.2$^+$ hybridomas were derived from a splenic population enriched for NK1.1$^+$ T cells from C57BL/6 mice. Spleen cells were first depleted of B cells by panning using rat polyclonal anti-mouse immunoglobulins (Ig) (DAKO A/S, Glostrup, Denmark). The nonadherent population was further depleted of CD8$^+$ and naive T cells using a MACS magnet (Miltenyi Biotec, Bergish, Germany). The cells were incubated with monoclonal antibodies (MoAbs) CD8α (YTC 169.4), CD8β (53.5.81) and Mel 14, followed by streptavidin microbeads (Miltenyi Biotec). 60%-80% of the remaining population was TCR$^+$CD4$^+$ and approximately 12% of all the cells were NK1.1$^+$TCR$^+$. The enriched population was stimulated either with plate-bound anti-CD3 Ab (hybridomas KT/7, KT/12, KT/22, KT/23) or with plate-bound anti-Vβ8.2 Ab (hybridoma Vβ/9), in the presence of IL-2 (supernatant from X-63 cells transfected to produce murine IL-2) or IL-2 and IL-7 (supernatant from J558 cells transfected to produce IL-7). After 4–5 days of culture the cells were fused with aminopterin-sensitive BW5147 αβTCR$^-$ thymoma cells using PEG 1500 and hybrids were selected in medium containing Hypoxantine-Aminopterin-Thymidine. For identification of the Vβ8.2$^+$ hybridomas, the cells were stained with Cychrome-conjugated anti-CD3ε Ab (Pharmingen San Diego, CA, USA) and with FITC-conjugated anti-Vβ8.2 (F23.2), which was purified and conjugated using standard procedures. DNA isolated from the Vβ8.2$^+$ hybridomas was analyzed for Vα14-Jα281 rearrangements by polymerase chain reaction (PCR) using the following primers: 5′ CTA AGC ACA GCA CGC TGC ACA 3′ (Vα14) and 5′ CAG GTA TGA CAA TCA GCT GAG TCC 3′(Jα281) essentially as described before [14]. Amplification was carried out by denaturation of DNA at 94 °C for 3 min followed by 30 cycles (94 °C 20 s, 60 °C 20 s, and 72 °C 30 s) in Taq buffer with 1.5 μM $MgCl_2$, 200 μM dNTP and 0.5 μM of each primer in a volume of 25 μl containing 2.5 U Taq polymerase.

Ceramides. The ceramides were synthesized and kindly provided by the Pharmaceutical Research Laboratory, Kirin Brewery Co., Ltd (Gunma, Japan). The ceramides were dissolved in 100% DMSO and kept as stock solutions at a concentration of 100 μg/ml. Before use, the stock solutions were diluted 100 times in phosphate buffered saline (PBS) and from this dilution the final concentration of 100 ng-3.12 ng/ml was added to the cultures. The tested ceramides were: αGalCer [(2S, 3S, 4R)-1-O-(α-D-galactopyranosyl)-N-hexacosanoyl-2-amino-1,3,4-octadecanetriol], α-glucosylceramide (αGlcCer) [(2S, 3S, 4R)-1-O-(α-D-glucopyranosyl)-N-hexacosanoyl-2-amino-1, 3, 4-octadecanetriol], β-galactosylceramide (βGalCer) [(2S, 3S, 4R)-1-O-(β-D-galactopyranosyl)-N-hexacosanoyl-2-amino-1, 3, 4-octadecanetriol], ceramide (Cer) [(2S, 3S, 4R)-N-hexaco-sanoyl-2-amino-1, 3, 4-octadecantriol], α-mannosylceramide (αManCer) [(2S, 3S, 4R)-2-amino-N-hexacosanoyl-1-O-(α-D-mannopyranosyl)-1, 3, 4-octandecanetriol], 3,4-deoxyα-galactosylceramide (3,4-deoxyαGalCer) [(2S)-1-O-(α-D-galactopyranosyl)-N-tetracosanoyl-2-amino-1-octadecanol], α-galactoso1–6αgalactosylceramide (Galα1–6Galα1–1′Cer) [(2S, 3S, 4R)-2-amino-1-O-(α-D-galactopyranosyl-(1–6)-α-D-galactopyranosyl)-N-hexacosanoyl-1, 3, 4-octadecanetriol], α-galactoso1–6αglucosylceramide (Galα1–6Glcα1–1′Cer) [(2S, 3S, 4R)-2-amino-1-O-(α-D-galactopyranosyl-(1–6)-α-D-glucopyranosyl)-N-hexacosanoyl-1, 3, 4-octadecanetriol], α-galactoso1–2α-galactosylceramide (Galα1–2Galα1–1′Cer) [(2S, 3S, 4R)-2-amino-1-O-(α-D-galactopyranosyl- (1–2)-α-D-galactopyranosyl)- N-[(R)-2-hydroxytetracosanoyl)]-1, 3, 4-octadecanetriol], β-galactoso1–3αgalactosylceramide (Galβ1–3Galα1–1′Cer) [(2S, 3S, 4R)-2-amino-1-O-(β-D-galactopyranosyl-(1–3)-α-D-galatopyranosyl)-N-[(R)-2-hydroxytetracosanoyl]-1, 3, 4-octadecanetriol], β-galactoso1–4βglucosylceramide (Galβ1–4Glcβ1–1′Cer) [(2S, 3S, 4E)-2-amino-1-O-(β-D-galactopyranosyl-(1–4)-β-D-glucopyranosyl)-N-hexacosanoyl-4-octadecene-1,3-diol], α-galactosylsphingosine (αGalSph) [(2S, 3S, 4R)-1-O-(α-D-galactopyranosyl)-N-acetyl-2-amino-1, 3, 4-octadecanetriol],

Table 1. CD1-restricted hybridoma cells used for analysis

Name	Vα	Vβ	Origin	Stimulation
VIII24	3.2	9	spleen	αCD3 Ab
XV19	ND*	ND	spleen	αCD3 Ab
XV104	4/5	8.3	spleen	αCD3 Ab
VIII57	2	5.1	spleen	αCD3 Ab
IIX82	10/11	ND	spleen	αCD3 Ab
KT/7	Vα14-Jα281	Vβ8.2	spleen	αCD3 Ab
KT/12	Vα14-Jα281	Vβ8.2	spleen	αCD3 Ab
KT/22	Vα14-Jα281	Vβ8.2	spleen	αCD3 Ab
KT/23	Vα14-Jα281	Vβ8.2	spleen	αCD3 Ab
Vβ/9	Vα14-Jα281	Vβ8.2	spleen	αVβ8.2 Ab

* ND-not determined.

aminobenzoylicα-galactosylcermide (ambenαGalCer) [(2S, 3S, 4R)-1-O-(α-D-galactopyranosyl)-N-(p-aminobenzoyl)-2-amino-1,3,4-octadecanetriol] [31].

Hybridoma stimulation assays. Hybridoma cells were cultured together with serial dilutions of CD1d-transfected and nontransfected cells (5×10^4–0.6×10^4) or with serial dilutions of splenocytes and thymocytes (4×10^5–0.5×10^5). As a positive control for T-cell hybridoma function the cells were stimulated with plate bound anti-CD3 Ab. For stimulation of hybridomas with the glycolipids, either A20 CD1 or RMA-S CD1 cells (1.25–5×10^4/well) were used as APC. For the titration of the different ceramides we used two-fold dilutions starting from 100 ng/ml down to 3.12 ng/ml. Where only one concentration was used, the hybridomas were stimulated with 100 ng/ml of compound. APC were pulsed with ceramide for 2 h and then the hybridomas (3×10^4/well) were added to the wells. Control culture contained hybridoma cells with CD1d transfected cells, nontransfected control cells or splenocytes without ceramides. After 24 h of incubation harvested supernatants were analyzed for IL-2 content in a CTLL assay. After 24–30 h of incubation CTLL were pulsed with 1μCi of [^{3}H]-thymidine per well for 4–12 h and the amount of incorporated radioactivity was counted in a liquid scintillation β-counter.

RESULTS

The two sets of CD1d-restricted hybridomas used

We have analyzed a panel of five hybridomas with combinations of different α- and β-TCR chains and five hybridomas using the restricted $V\alpha14^+V\beta8.2^+$ TCR for reactivity to ceramide-based ligands presented on CD1d. The first group consists of five hybridomas obtained from the $CD4^+$ T-cell population found in the spleen of MHC class II-deficient mice. They were shown to be CD1d-reactive and became activated when cultured with syngeneic splenocytes alone [15]. As shown in the upper panel of Table 1 (and data not shown) none of these hybridomas utilize $V\alpha14^+$ or $V\beta8.2^+$ TCR chains. The second group of cells presented in the lower panel of Table 1 were derived from enriched population of splenic NKT cells from C57BL/6 mice and stimulated by plate bound Ab to CD3 or Vβ8.2 in the presence of cytokines. Individual hybridomas were screened for the expression of Vβ8.2 using flow cytometry and for Vα14-Jα281 rearrangements by PCR. Five positive hybridomas were selected for further analysis and are referred to as $V\alpha14^+$ hybridomas below.

Heterogeneity of reactivity by hybridomas expressing diverse TCR

Previous experiments had demonstrated differences in the patterns of reactivity within the set of CD1d-reactive hybridomas with diverse TCR [15, 37]. To verify this, the hybridomas were challenged with three types of CD1d-transfectants: A20 CD1d, RMA-S CD1d, and L-CD1d cells (referred further as RMA-S CD1, A20 CD1, L-CD1), and with splenocytes and thymocytes from C57BL/6 mice. Compiled results from several experiments, presented in Table 2 and exemplified in Fig. 1, demonstrate that there was a difference in the reactivity between the hybridomas to the various types of APC. Firstly only the VII57 hybridoma responded stronger to the thymocytes than to the splenocytes, while the rest of the hybridomas responded preferentially to splenocytes, although XV104 cells showed a very low response

Table 2. Summary of the reactivities of hybridomas with diverse T-cell receptor*

	Rank of order of stimulator cells				
Hybridoma	1	2	3	4	5
XV19	A20 CD1	Spleen	Thymus	L-CD1	RMA-S CD1
VIII24	A20 CD1	Spleen	Thymus	L-CD1	RMA-S CD1
XV104	L-CD1	Spleen	Thymus	A20 CD1	RMA-S CD1
VII57	Thymus	Spleen	= RMA-S CD1	L-CD1	A20 CD1
IIX82	Thymus	= Spleen	RMA-S CD1	=Ll-CD1	A20 CD1

* Stimulators are ranked in decreasing potency from left to right. The most potent is 1. =; indicates equal response.

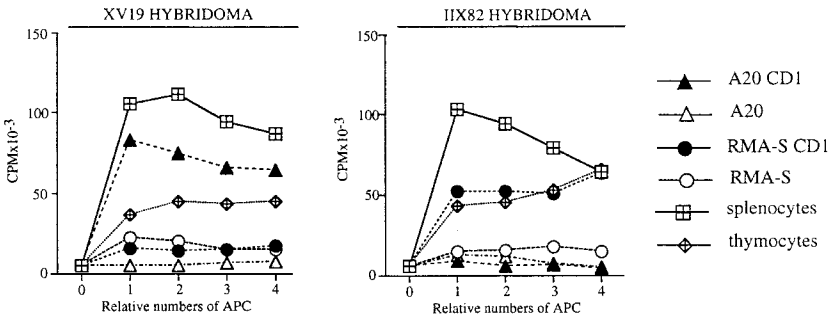

Fig. 1. Reactivity of XV19 and IIX82 hybridoma to different types of CD1d expressing cells. The hybridoma cells (3×10^4/well) were cultured together with serial dilutions of splenocytes and thymocytes (0.5–4×10^5/well) or with CD1d-transfectants (0.6–5×10^4/well). After 24 h, harvested supernatants were examined for interleukin (IL)-2 content in a CTLL assay. The relative numbers indicate a two-fold increase of the concentration of antigen-presenting cells (APC). The data are representative of two to five experiments.

to both types of cells. Secondly, the hybridomas showed different preference for the three CD1d-transfectants (Fig. 1 and Table 2). As shown in Fig. 2, transfected RMA-S and A20 cells expressed similar levels of CD1d, nontransfected A20 cells were negative for CD1d, while RMA-S expressed low levels of CD1d. CD1-transfected L cells displayed CD1d levels comparable to A20 and RMA-S transfectants (data not shown). For the analysis of reactivity to exogenously added glycolipids presented on CD1d, we selected for each hybridoma a CD1-transfectant expressing high levels of CD1d, but which did not induce a strong hybridoma response in the absence of added ligand.

Analysis of glycosylceramide reactivity by CD1d-restricted hybridomas with diverse TCR

When the $V\alpha14^+$ hybridomas were stimulated we noted that there was a difference in the strength of response when αGalCer was loaded on RMA-S CD1 versus A20 CD1 cells. After stimulation with A20 CD1 cells loaded with αGalCer the response of two tested hybridoma cells (KT/7 and KT/23) was approximately 10 times stronger than when αGalCer had been loaded on RMA-S CD1 cells. Figure 3 shows the data for the KT/7 hybridoma. Longer incubation of αGalCer with RMA-S CD1 before adding

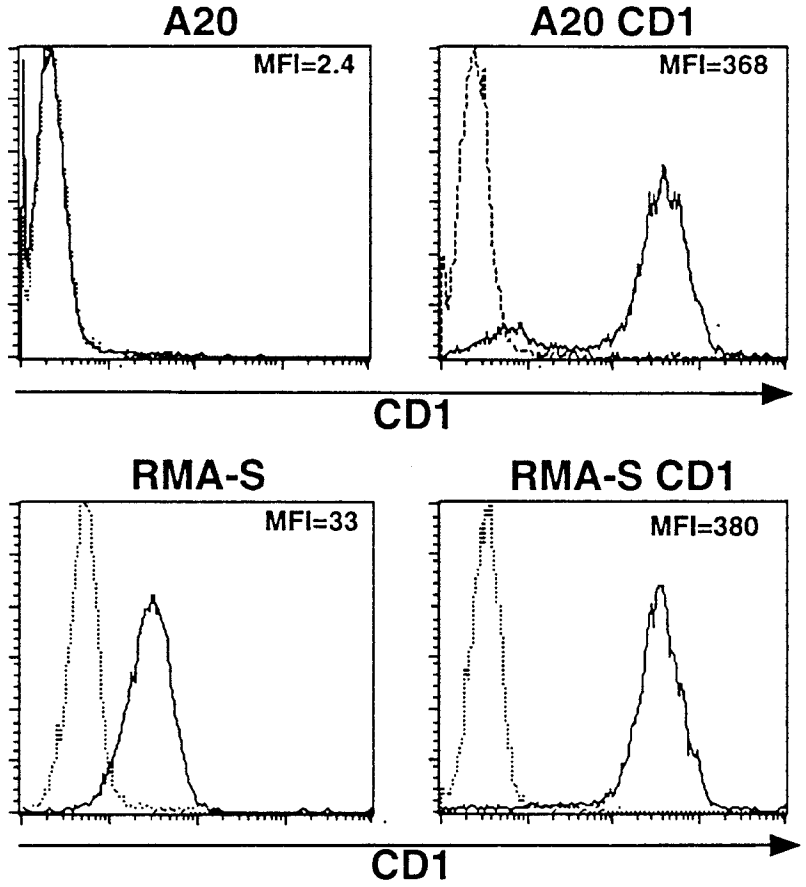

Fig. 2. Expression of the CD1d molecule on CD1d-transfected and nontransfected A20 and RMA-S cells. The cells were stained for CD1d (solid line), the background staining is

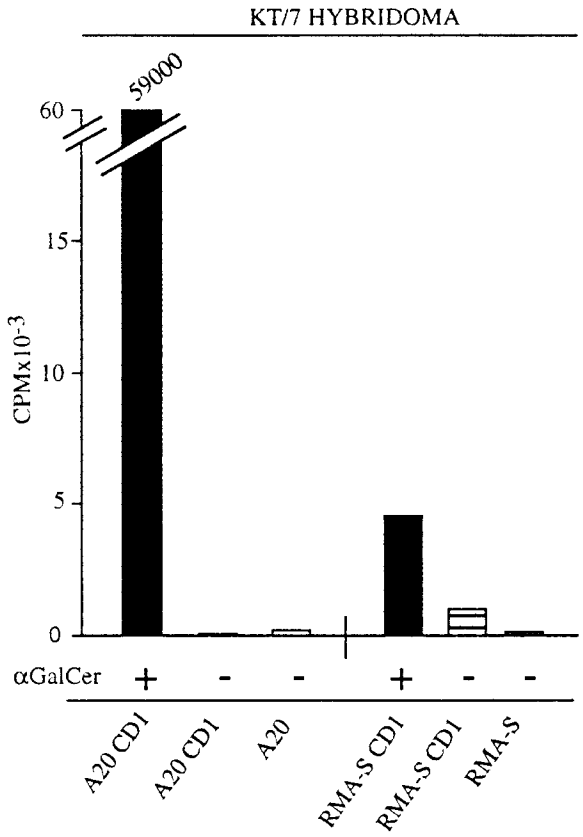

Fig. 3. Comparison of the hybridoma response to αGalCer loaded on different types of APC. A20 CD1d and RMA-S CD1d cells (2.5×10^4/well) were pulsed for 2 h with 100 ng/ml of αGalCer before the hybridoma cells were added to the cultures. After 24 h harvested supernatants were examined for IL-2 content in a CTLL assay. The response of KT/7 hybridoma to αGalCer loaded on A20 CD1d and RMA-S CD1d is indicated in black bars. The hybridoma was also cultured with CD1d-transfectant (striped bars) and nontransfected cells (white bars) alone. The data are representative of three experiments.

the hybridoma cells did not increase the strength of the hybridoma response (data not shown). As shown above (Fig. 2), the observed result could not be explained by differences in the levels of CD1d expression on the transfectants. We decided therefore to use preferentially A20 CD1 cells for presentation of the differently glycosylated ceramides to the hybridomas with TCR diverse. However, owing to high reactivity of XV19 and VIII24 hybridomas to A20 CD1 cells, some of the experiments for these cells were performed using RMA-S CD1 transfectants as APC. The hybridomas were stimulated with CD1d-transfectants preincubated with the panel of ceramides including variants with single or double carbohydrate residues, that had two different anomeric configurations (α or β). None of the analyzed hybridomas with diverse TCR showed any detectable reactivity to the tested glycolipids (Table 3).

Table 3. Responsiveness of CD1-reactive hybridomas with diverse TCR to the ceramides presented on CD1-transfectants*

Ligand	XV19	VIII24	VII57	IIX82	XV104
Ceramide	–†	–†	–	–	–
βGalCer	–†	–†	–	–	–
αGalCer	–†	–†	–	–	–
αGlcCer	–†	–†	–	–	–
αManCer	–†	–†	–	–	–
3,4deoxy αGalCer	–†	–†	–	–	–
αGalSph	–†	–†	–	–	–
Galα1–6Galα1–1′Cer	ND‡	–	–	–	–
Galα1–6Glcα1–1′Cer	ND	–	–	–	–
Galα1–2Galα1–1′Cer	ND	–	–	–	–
Galβ1–3Galα1–1′Cer	ND	–	–	–	–
Galβ1–4Glcβ1–1′Cer	ND	–	–	–	–

*Data are representative of at least two experiments and were performed with A20 CD1 cells, except where indicated. †Experiment was performed with RMA-S CD1 as APC. ‡ND; not determined.

Analysis of reactivity of the Vα14$^+$ hybridomas

As anticipated from previously published data [31], the five Vα14$^+$ hybridomas were able to recognize CD1d-transfected cells pulsed with αGalCer (Fig. 4A). Further, characteristic features of NKT cells are autoreactivity and responsiveness to CD1d-transfected cell lines in the absence of added ligands [2, 37]. We analyzed the Vα14$^+$ hybridomas for responsiveness to different types of CD1d-expressing stimulators as well as to splenocytes and thymocytes from C57BL/6 mice. The five hybridomas displayed similar reactivities (shown for one representative hybridoma in Fig. 4), i.e. only low reactivity to syngeneic splenocytes, absence of reactivity to syngeneic thymocytes (Fig. 4B) and very low reactivity to the CD1d-transfected A20, RMA-S or L-cells (Fig. 4C).

The ligand-fine specificity can be heterogeneous also between the Vα14$^+$ hybridomas, despite the high similarity of TCR [34]. We therefore investigated the reactivities of the five generated Vα14$^+$ hybridomas. The cells were stimulated with serial dilutions of the differently glycosylated variants of glycolipids presented on A20 CD1 cells (Fig. 5). Our results showed similar responses by all five hybridomas (compiled in Table 4), and were essentially consistent with data published before describing the reactivity of cells using one specific Vα14-Jα281/Vβ8.2 TCR [31]. The Vα14$^+$ cells did not recognize βGalCer, Cer, βGal1–4Glcβ1–1′Cer and ambenαGalSph and displayed only a low reactivity to Galα1–6Galα1–1′Cer and Galα1–6Glcα1–1′Cer. In our hands, αGlcCer and Galα1–2Galα1–1Cer induced a similar or stronger response as compared to αGalCer. Figure 5 shows representative results from a stimulation of KT/23 cells. Further, all five Vα14$^+$ hybridomas responded well to the variant of αGalCer with a truncated fatty-acyl chain, αGalSph (Table 4). We also observed that Galβ1–3Galα1–1′Cer, a ceramide with

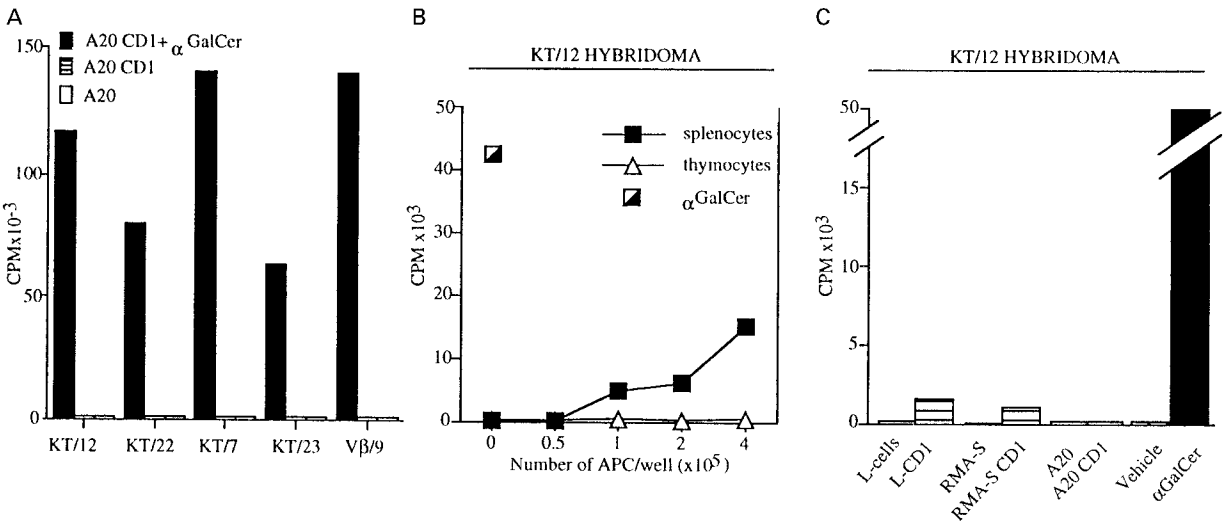

Fig. 4. The Vα14$^+$ hybridomas responded to αGalCer. (A) αGalCer (100 ng/ml) was preincubated on A20 CD1d cells (5×10^4/well) before the hybridoma cells were added to the culture. In parallel the hybridomas were cultured with CD1d-transfected and nontransfected cells in the absence of glycolipid. The data are representative of at least five experiments. (B) KT/12 hybridoma cells were cultured with serial dilution ($0.5–4 \times 10^5$/well) of B6 splenocytes and thymocytes. For comparison, the cells were cultured with A20 CD1d cells pulsed with 100 ng/ml of αGalCer. (C) KT/12 cells were cultured with A20 CD1d, RMA-S CD1d and L-CD1d cells (5×10^4/well). The response to the CD1d-transfectants was compared with the response to αGalCer preloaded on A20 CD1d cells. The results are representative of at least two experiments.

the distal sugar in β anomeric configuration did not induce any detectable hybridoma response (Fig. 5 and Table 4). Thus, the five Vα14$^+$ hybridomas showed essentially identical patterns of reactivity both to CD1d-expressing APC, and to the tested ceramides presented on CD1d.

DISCUSSION

In this report we have studied hybridomas representative of the two subsets of NKT cells, characterized by utilization of a diverse and restricted repertoire of TCR, respectively. The cells were analyzed for their reactivities to different APC expressing CD1d and to glycosylceramides presented on murine CD1d. The hybridomas with diverse TCR showed

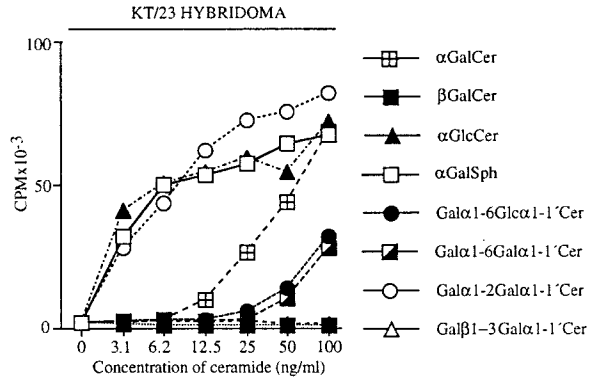

Fig. 5. Responsiveness of the Vα14$^+$ hybridoma KT/23 to the panel of differently glycosylated ceramides. The KT/23 cells were stimulated with the ceramides at the indicated concentrations. Harvested supernatants were examined for IL-2 content in a CTLL assay. The data are representative of two to four experiments, αGalCer was tested more than four times.

Table 4. Responsiveness of the Vα14$^+$ hybridomas to ceramides presented on A20 CD1.1 cells*

Ligand	KT/7	KT/12	KT/22	KT/23	Vβ/9
Ceramide	−†	−	−	−	−
αGalCer	++	++	++	++	++
βGalCer	−	−	−	−	−
αGlcCer	+++	+++	+++	+++	+++
αManCer	−	−	−	−	−
3,4deoxyαGalCer	−	−	−	−	−
αGalSph	+++	+++	+++	+++	+++
Galα1–6Galα1–1′Cer	+	+	−	+	+
Galα1–6Glcα1–1′Cer	+	+	−	+	+
Galα1–2Galα1–1′Cer	+++	+++	+++	+++	+++
Galβ1–3Galα1–1′Cer	−	−	−	−	−
Galβ1–4Glcβ1–1′Cer	−	−	−	−	−

*Results are a compilation of two to four experiments, αGalCer was tested more than four times.

†(+) low response (++) intermediate response (+++) high response (−) negative response.

different preferences in the reactivity to syngeneic splenocytes, thymocytes and to CD1d-transfected cell lines. These data indicated heterogeneity in the recognition of ligands within this population, that was in agreement with previous findings [15, 36, 37]. We also analyzed the reactivity of hybridomas with restricted ($V\alpha14^+V\beta8.2^+$) TCR to CD1d-expressing cells in the absence of exogenous ligands. The $V\alpha14^+$ hybridomas gave only moderate responses to splenocytes, and no, or very low, responses to syngeneic thymocytes or the tested CD1d-transfectants. This is somewhat in contrast to results obtained by others [2], but it should be noted that the hybridomas presented here were selected only by the type of TCR, not by their reactivity, thus auto- or direct CD1d-reactivity may not be a feature of all $V\alpha14^+$ NKT cells.

NKT cells with restricted ($V\alpha14^+V\beta8.2^+$) TCR have been shown to respond to αGalCer and some variants of this glycolipid when presented on CD1d. NKT cells, with diverse and restricted TCR have been demonstrated to be CD1d-reactive. Because CD1 shows very low polymorphism and is well conserved, we reasoned that CD1d might present a restricted set of ligands and thus that the two CD1d-restricted T-cell subsets might recognize similar antigens. We examined the five hybridomas with diverse TCR for their reactivity to differently glycosylated ceramides. Following incubation with CD1d-transfected cells, none of the tested glycolipid variants were able to induce an increased response by any of the hybridomas. Similarly to previously published data [31], the generated $V\alpha14^+$ hybridomas responded to αGalCer and selected variants of that glycolipid. Further, all five $V\alpha14^+$ hybridomas displayed essentially identical patterns of reactivity suggesting that their TCR Vβ8.2 chains may be similar. Only one hybridoma, KT/22, did not react to two variants of glycolipid with double sugar moiety (Galα1–6Glcα1–1′Cer and Galα1–6Galα1–1′Cer), which generated a weak response by the four remaining $V\alpha14^+$ hybridoma cells. It is possible that this minor dissimilarity reflects a quantitative rather than qualitative difference.

While αGalCer has been found to be the most potent among the glycolipids to stimulate $V\alpha14^+$ NKT cells in a different system, we found that other variants (αGlcCer, αGalSph and Galα1–2Galα1–1′Cer) could be equally or more potent in inducing a response of the $V\alpha14^+$ hybridomas tested here. Notably, all five hybridomas responded to αGalSph, a αGalCer with a truncated fatty acyl chain. This finding differs from that of a previous report [31], but is in line with those of recent studies performed by Naidenko *et al.* showing that biotinylation of αGalCer, which replaces most of the fatty acyl chain, decreases, but does not abrogate the response of a $V\alpha14^+V\beta8.2^+$ hybridoma cells [38]. Possible reasons for the apparent discrepancy between these investigations could be owing to differences in the employed experimental systems. The three studies used independently generated $V\alpha14^+$ T cells, that are likely to have dissimilar TCR Vβ8.2 rearrangements. Indeed, a recent investigation by Gumperz and coworkers demonstrated that two independent Vα14-Jα281/Vβ8.2 hybridomas could have very different patterns of reactivity to lipid ligands, possibly owing to the TCR-β chains [34]. Further, the previous report was unable to detect αGalSph reactivity [31], and analyzed the proliferation of primary T lymphocytes expressing a transgenic Vα14-Jα281/Vβ8.2 TCR in response to glycolipids presented on splenic dendritic cells. In contrast, Naidenko *et al.* tested responses of T-cell hybridomas using CD1d-transfected APC, as it was done in present study. Thus, the different assay systems used may have different sensitivities to CD1d-presented ligands.

We found that αGalCer presented on A20CD1 cells stimulated the $V\alpha14^+$ hybridomas more efficiently than when loaded on RMA-S CD1 cells, despite the fact that two transfectants had similar levels of CD1d. The explanation might be a difference in the antigen-presenting mechanisms between T cell-(RMA-S) and B cell-(A20) derived cell lines. Kawano and colleagues showed that chloroquine and concanamycin A, that prevent acidification of and transportation to late endosomes, respectively, could inhibit presentation of αGalCer by dendritic cells [31], thus indicating a requirement for endosomal processing pathways. Further, CD1 has been found preferentially localized to the acidic endosomal compartments, including MHC class II-containing compartments (MIIC) [39, 40]. Because B cells but not T cells express MHC class II, the presentation of exogenous αGalCer on A20CD1 transfectants is expected to be more efficient. It is also possible that differences in the expression pattern of costimulatory molecules on the APC could influence the reactivity of hybridomas to αGalCer.

Several ceramide-based structures, but not αGalCer itself, have been detected in mammalian tissues [41, 42] and also in multidrug-resistant human tumour and cancer cell lines [43]. The possibility remains open that such endogenous compounds, involved in cell proliferation, differentiation, and oncogenic transformation, makes up a group of potential ligands for NKT cells. Recently, murine CD1d-restricted $V\alpha14^+$ hybridomas have been shown to recognize phospholipids structures, i.e. purified phosphatidylinositol (PI), purified distearoyl phosphatidylethanolamine (PE) and synthetic dipalmitoyl phosphatidylglycerol (PG), that may represent a group of potential self ligands recognized by CD1d-reactive T cells. Further, the same set of ligands could be recognized by some, but not all (e.g. not by XV19, VIII24 and XV104), hybridomas with diverse TCR [34].

The GPI has been shown to be a natural ligand associated with murine CD1d [30]. In mammals, many of the surface membrane enzymes, receptors, complement defense proteins, blood-group antigens, and molecules required for cell adhesion and activation [44] have been found to be GPI-anchored [45]. Additionally, there are many GPI-linked protozoan surface macromolecules. Recently it was shown that NKT cells function as T-helper cells in the antibody response directed against GPI-linked parasitic antigens [13], demonstrating that exogenous forms of protozoan GPI are targeted by NKT cells. These findings may indicate that different forms of GPI can bind and be recognized by CD1-reactive T cells, especially that the core structure of GPI is conserved between mammals and microbes [45]. NKT cells are also involved in the defense against bacterial infection [10, 11].

Although bacterial lipids have not been shown to stimulate CD1d-restricted T cells, the structures of mycobacterial antigens recognized by human CD1b-restricted T cells are very similar to mammalian GPI [27]. Because CD1, in contrast to MHC class I molecules, is not highly polymorphic, it is possible that different types of antigens could bind to CD1 via a similar lipid motif. The variations in the carbohydrate moiety could account for the differences in the recognition of various ligands by CD1d-restricted T cells. The results from these studies on the synthetic ligands presented on CD1d could be useful for further investigations of the cellular compounds recognized by CD1-restricted T cells.

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